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Fast and sensitive detection of mycotoxins in wheat using microfluidics based Real-time Electrochemical Profiling

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ABSTRACT

The objective of the study has been the development of a new sensing platform, called Real-time Electrochemical Profiling (REP) that relies on real-time electrochemical immunoassay detection. The proposed REP platform consists of new electrode arrays that are easy to fabricate, has a small imprint allowing microfluidic system integration, enables multiplexed amperometric measurements and performs well in terms of electrochemical immunoassay detection as shown through the deoxynivalenol detection assays. The deoxynivalenol detection has been conducted according to an optimised REP assay protocol using deoxynivalenol standards at varying concentrations and a standard curve was obtained ($y=-20.33\ln(x)+124.06$; $R^2=0.97$) with a limit of detection of 6.25 ng/ml. As both ELISA and REP detection methods use horse radish peroxidase as the label and 3.3′,5.5′-Tetramethylbenzidine as the substrate, the performance of the REP platform as an ELISA reader has also been investigated and a perfect correlation between the deoxynivalenol concentration and the current response was obtained ($y=-14.56\ln(x)+101.02$; $R^2=0.99$). The calibration curves of both assays have been compared to conventional ELISA tests for confirmation. After assay optimisation using toxin spiked buffer, the deoxynivalenol detection assay has also been performed to detect toxins in wheat grain.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by filamentous fungi growing on many plants and processed food, either in the field or during storage. Mycotoxins are carcinogenic, produced quickly in damp conditions and can be fatal in high doses; therefore, they have been considered as the most significant chronic dietary risk factor, even higher than synthetic contaminants, food additives or pesticide residues. Deoxynivalenol (DON), as one of the mycotoxins, is economically very important due to its prevalence and heat stability. DON persists in grains during storage and is unaffected by high temperature and pressure during processing (Wolf-Hall et al., 1999). To avoid consumers' health risk due to unacceptably high dietary intake of Fusarium toxins, the EC has established maximum levels for DON (1.25 ppm in cereals; 0.2 ppm in processed cereal-based foods and baby foods) (European Commission, 2006; Lancova et al., 2008). For this reason, DON testing has a great importance to prevent contaminated food to reach the consumer as well as to be able to import the food products by passing the regulations set by the countries.

Traditional mycotoxin tests utilise gas chromotography, mass spectrometry, liquid chromatography or enzyme-linked immunosorbent assay (ELISA) type assays that use expensive equipment, need a specialist and have time consuming procedures. These tests also require sample transportation from storage to the laboratories that cause increased waiting time and high costs. Therefore, there is a need to develop a detection system that is cheap, quick to process, uses low sample and reagent volume and easily operated. Recent advances in the area of sensor technology and lab-on-a-chip applications have enabled the miniaturisation of the devices and multiplex testing of a range of analytes. Therefore, biosensor technology has the potential for the manufacture of mycotoxin detection devices (Tothill, 2011; Vidal et al., 2013). Recent advances in the area of sensor technology have enabled the miniaturisation of the devices and multiplex testing of a range of analytes. Therefore, biosensor technology has the potential for the manufacture of testing devices for toxin analysis in crops as the global food safety testing market by contaminants is estimated to

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grow at an annual growth rate of 10.46% to $2.5 billion in 2015 (MarketPublishers, 2011).

Recently, mycotoxin testing has been performed using various biosensing techniques from simpler immunodipstick type tests to the label free quartz crystal microbalance (QCM) (Vidal et al., 2009) and surface plasmon resonance (SPR) (Li et al., 2012; Choi et al., 2011), and electrochemical tests (Khan et al., 2011; Hervas et al., 2011; Rene Perrotta et al., 2011; Vidal et al., 2012). In a recent paper, Lattanzio et al. (2012) described a multiplex dipstick immunoassay that provided simultaneous detection of T-2 and HT-2 toxins, DON and fumonisins in crops. The results obtained from this study indicated that the optimised immunoassay was able to detect target mycotoxins at cut-off levels equal to 80% of EU maximum permitted levels. Kolosova et al. (2008) developed a lateral-flow immunoassay using a colloidal gold-labelled monoclonal antibody for the qualitative rapid determination of DON. While immunodipstick tests do not require an expert for use and they provide rapid, inexpensive testing, their main short-coming is their limited sensitivity and in most cases they can be used as a qualitative screening tool complementary to more accurate techniques. Commercial strip-tests are currently available for DON and aflatoxin detection in grain that can be used either as semi-quantitative or quantitative tests.

High sensitivity, selectivity, rapid analysis, ability to operate in turbid solutions and the possibility of miniaturisation enabled electrochemical biosensors to become the most commonly used biosensors over other technologies (Shah and Wilkins, 2003). For example, Heurich et al. (2011) developed a disposable electrochemical immunosensor using screen printed electrodes for ochratoxin A detection in wine and obtained a limit of detection of 0.05 μg/L with a linear dynamic detection range of 0.01–100 μg/L. Hervas et al. (2009) have improved the electrochemical immunoassay response by means of magnetic nanoparticles and obtained a detection limit (LOD) of 0.011 μg/L for the zearalenone in baby food samples. In the literature several other studies can be found related with mycotoxin detection using electrochemical techniques (Bone et al., 2010; Asuncion Alonso-Lomillo et al., 2011; Barthelmebs et al., 2011; Panini et al., 2011) ; however, most of these publications describe the results of the tests that are performed using bench top potentiostats with off the shelf electrodes and require hands on work. This shows that most of the publications describe only the results for proof of principle of the toxin detection using biosensors and the number of studies performed using integrated systems with the necessary fluidics is quite limited. In addition mostly, the assay procedures are long, labourous and not suitable for automation. In response to that, we have developed a new detection technology, which we term Real-time Electrochemical Profiling (REP™). While this technology relies on the fundamental basics of electrochemical immunosensing, in particular amperometry, it has several key features including a new electrode array, microfluidics based assay and real-time amperometric measurements during the flow of enzyme substrate. For the common flow injection electrochemical immunoassay platforms, initially in a separate container or fluidic system the enzyme immunoassay is performed, and later the reaction solution is sent to (usually) a bare electrode for the detection at a certain flow rate. However with the REP system, we were able to combine both steps together on one electrode surface. That distinguishes our sensing platform from other flow injection based electrochemical immunoassays. In addition, whole assay is performed during the reagents flow; therefore, there is no need to stop the flow either during the analyte binding or enzymatic reaction; hence the sensing can be achieved reasonably fast. In an earlier study, a new electrode array and integrated microfluidics have been designed and characterised in order to create a sensor chip that is not only easy, rapid and cheaper to produce but also have a smaller imprint and good electrochemical sensing properties (Uludag et al., 2014). In the current study, this new electrode array, comprising Au quasi-reference electrode and shared reference/counter-electrodes with the integrated microfluidics, has been used for the detection of DON with REP platform.

2. Materials and instrumentation

Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) tablets, mercaptoethanol, mercaptoundecanoic acid (MUDA), ethanolamine, spectrophotometric grade ethanol, horse radish peroxidase (HRP), 3.3′,5.5′-Tetramethylbenzidine (TMB) ready to use reagent (contains H_2O_2), hydrochloric acid (HCl), N-hydroxysuccinimide (NHS), and potassium ferrocyanide were purchased from Sigma-Aldrich (Poole, UK). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and Protein A was purchased from Thermo Scientific (Istanbul, Turkey). Deoxynivalenol (DON) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). DON–HRP conjugate and AgraQuant deoxynivalenol test kit were obtained from Romer Labs (Tulln, Austria). DON antibody was purchased from Lifespan Biosciences (Seattle, USA). Potassium chloride (KCl) and biotin were purchased from Fisher Scientific (Loughborough, UK). Oxygen free argon was purchased from Habas (Istanbul, Turkey). Ultrapure water (18 MΩ cm^{-1}) was obtained from a Milli-Q water system (Millipore Corp., Tokyo, Japan).

2.1. Safety awareness

Due to the toxic properties of DON, safety precautions were applied, such as wearing gloves, protection glasses and laboratory coat at all times and a facial mask when handling powder DON. The toxin was stored in a locked fridge specified for toxic reagents according to safety instructions.

3. Methods

3.1. Transducer fabrication

A sensor chip has been designed and fabricated on silicon dioxide wafer that consists of 2 sets of electrode arrays; each set consists of 3 working electrodes (d=1 mm) with shared Au counter and quasi-reference electrodes (Fig. 1A). The design of the electrodes was formed on the silicon dioxide wafer by means of a Fine Metal Mask made of a laser cut patterned stainless steel, and Au metal was deposited on the wafer using an electron beam evaporator. Before the application of Au (200 nm), a 20 nm Ti layer is applied on to the silicon dioxide wafer as an intermediary adhesive layer to increase the adhesion between the Au and silicon dioxide wafer. After metal evaporation, the silicon wafer has been cut into 10×20 mm^2 pieces that form the sensor chips with two electrode arrays (Fig. 1A). A sensor cassette has been designed and fabricated from poly(methyl methacrylate) (PMMA) and a double sided sticky tape formed a microfluidic channel ($\sim$ 10 μL) on the electrode arrays (Fig. 1A and B). A potentiostat (MicroStat 8000; Dropsens, Astuias, Spain), a syringe pump (Mitos Duo XS; Dolomite, Royston, UK), an injection valve and a sensor chip docking station (Fig. 1C) have been set up for the experimental work.

3.2. Electrochemical analysis

Cyclic voltammetry and amperometric measurements were performed with a MicroStat 8000 Electrochemical Analyzer with